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(54) Title: PHOSPHORYLATING REAGENTS

(57) Abstract

There is provided a phosphorylating reagent for phosphrylation of amino acids or compounds formed therefrom. The phosphorylating reagent is of utility in solution or solid-phase chemistry, and particularly for the solid-phase synthesis of phosphorylated peptides and combinational libraries of phosphorylated organic compounds. Also provided for is a method of phosphorylating oxygen, nitrogen and sulphur nucleophides, for example amino acid and peptides.

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Phosphorylating Reagents

This invention describes novel phosphorylating reagents and the use thereof for solution or solid-phase chemistry and their particular use for the solid-phase synthesis of phosphorylated peptides and combinatorial libraries of phosphorylated organic compounds.

The reversible phosphorylation of proteins on serine, threonine and tyrosine residues, as catalysed by protein kinases and phosphatases, is the principal mechanism by which eukaryotic cells (the cells of multicellular organisms) respond to external stimuli[1,2]. Three groups of enzymes referred to collectively as the protein phosphatases (these enzymes hydrolyse the phosphoryl group of a phosphoprotein) are responsible for the dephosphorylation of the phosphoproteins. One group known as the serine-threonine protein phosphatases are collectively responsible for the dephosphorylation of certain phosphorylated serine or threonine residues within phosphoproteins (see Fig. 1A) and several different types exist (e.g. PP1, PP2A, PP2B and PP2C) most of which appear to be associated with regulatory proteins. A second group are referred to as the protein tyrosine phosphatases and these enzymes

hydrolytically remove the phosphoryl group from certain phosphotyrosine residues within phosphoproteins, Fig. 1B. The third group of enzymes is responsible for removing the phosphoryl group from phosphohistidine residues within phosphoproteins, Fig. 1C.

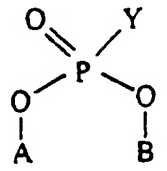
Structure-activity studies for the phosphorylated peptide substrates of protein phosphatases have been limited by the availability of structurally diverse substrates because, to date, almost all of these have been prepared by enzymic phosphorylation using adenosine triphosphate (ATP) and appropriate protein kinase enzymes which are specific for certain sequences[3]. The specific nature of these enzymes restricts the scope of these studies in addition to the extra complications of separating the products of the reaction e.g. separating the phosphorylated peptide from adenosine monophosphate (AMP). Moreover, non-enzymic syntheses of phosphopeptides, in particular phosphothreonine peptides is severely hampered by β-elimination of phosphoric acid diester which occurs in synthetic intermediates to give the corresponding dehydroamino acid moieties[4-6]. Phosphothreonine peptide syntheses typically employ large excesses of highly electrophilic phosphorus (III) reagents to introduce phosphorus into the preformed peptide and then an oxidant (e.g.tertiary-butyl hydroperoxide) is required to convert the phosphite triester to the phosphate triester prior to deprotection of the ester groups[4,5]. While the peptide exists as its phosphate triester, it is particularly vulnerable to β-elimination, which is undesirable.

The existing methods for avoiding β-elimination in the synthesis of phosphoserine and phosphothreonine peptides involve introducing each of the phosphorylated

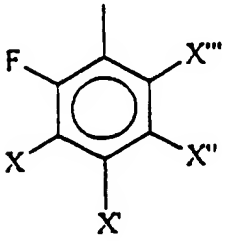
amino acid residues as their protected phosphate diester monoanions[6] . These are however tedious to prepare.

It is an object of the present invention to provide a phosphorylating agent that would be electrophilic enough to react directly and rapidly with primary and secondary alcohol groups within resin-bound peptides. Such agents would obviate the need for an oxidant, and could possess labile phosphate ester protecting groups that would be compatible with solid-phase peptide synthesis.

This invention provides an electrophilic phosphorylating reagent for amino acids and/or peptide sequences thereof comprising a compound as represented by formula (I):



(I)



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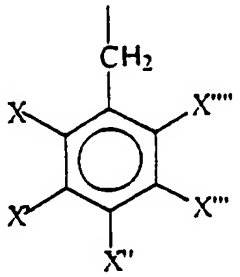

(III)

wherein: A is a substituted aromatic group which is represented by formula (II) e.g. a fluorophenyl or A is an acid cleavable functionality such as a benzyl or substituted benzyl group represented by formula (III); B is a substituted aromatic group represented by formula (II) e.g. a fluorophenyl group, but not a

benzyl or a substituted benzyl group;
each X, X', X'', X''' and X'''' are individually H or F atoms or any suitable moiety;
Y is any halogen or leaving group.

The leaving group Y is the group which does not contain the phosphorous atom following cleavage of compound I; for example Y can be Cl, Br, I, -NRR'R''(as a quaternary ammonium salt), -OR, -SR (wherein each group R, R' or R" is any group which does not affect the lability of the leaving group Y, especially under acidic and/or basic conditions, for example R, R' or R" can individually be -H, $-CH_3$, $-C_2H_5$, $-C_6H_5$, $-C(O)-C_{1-12}$ or $-CH=C(OH)-C_{1-12}$).

The reference to "any suitable moiety" with regard to groups X, X', X'', X''' and X'''' refers to any atom or group thereof which does not affect the lability of the compounds represented by formulae II or III.

In one embodiment, the reagent is bis(tetrafluorophenyl) chlorophosphate; and in this embodiment the preferred reagent is bis (2, 3, 5, 6-tetrafluorophenyl) chlorophosphate.

In a further embodiment, the reagent is a benzyl, fluorophenyl halophosphate; and in this embodiment the preferred reagent is a benzyl, polyfluorophenyl chlorophosphate.

It can be seen that reagents represented by the formula (I) have a fluorine in at least one *ortho* position on at least one of the aromatic rings wherein the remaining positions X, X', X'' and X''' on (II) and X,

X', X'', X''' and X'''' on (III) can each be -H or -F atoms or any suitable moiety in any permutation.

Additionally, the -H atom or -F atom or suitable moiety may be in the presence or absence of one or more similar or dissimilar other ring substituents.

A further embodiment has a halogen or other leaving group attached to the phosphorus atom of reagent (I) at Y, where the leaving group can be one of -OR, -NRR', -NRR'R'' or -SR, wherein R, R' and R'' can be any suitable moiety.

The invention further provides a method for the phosphorylation of oxygen, nitrogen or sulphur nucleophiles of amino acids and/or peptides wherein the nucleophile is treated with an excess of a reagent of general formula (I) followed by hydrolysis of the product.

Preferably the hydrolysis reagent is trifluoroacetic acid.

The oxygen nucleophile may be part of a primary or secondary alcohol, phenol, carboxylate or enolate group.

The amino acids may be present as single species or in combination within or outwith the same molecule, as in peptide sequences.

Suitably, the amino acid(s) may be tyrosine, serine and threonine.

In one particular embodiment of the invention, the amino acid is present as a resin bound moiety.

In further embodiments of the invention, the

phosphorylation method may be utilised in solid, liquid or gel phase.

The method is of considerable potential in the solid-phase synthesis of a whole range of organic phosphates from primary and secondary alcohols and phenols and is completely compatible with combinatorial and permutational organic synthesis.

In the area of peptide chemistry the method offers very significant advantages over the previously used two step phosphitylation-oxidation strategies, furthermore, the use of bis-(pentafluorophenyl) chlorophosphate (11) is of particular utility in the preparation of peptides containing two or more phosphorylated residues via a "global phosphorylation" strategy which involves introducing all of the phosphoryl groups in one step after the synthesis of the required peptide. The same is true for the introduction of more than one phosphoryl group into other organic molecules which contain more that one alcohol and/or phenol group.

The examples illustrate that primary alcohols, secondary alcohols and phenols whether present as single species, or in combination within or outwith the same molecule, are efficiently phosphorylated by the polyfluoroaromatic chlorophosphate reagents. Other oxygen nucleophiles, for example, carboxylate and enolate, and other nucleophiles, for example, those derived from nitrogen and sulphur are also expected to react with similar efficiency with the reagent.

The examples herein relate to the phosphorylation reaction by bis-(pentafluorophenyl chlorophosphate (11) and other polyfluoroaromatic halophosphates shown by

general formula I, where any, some or all X groups is H and/or F or other suitable moiety in any permutation whether in the presence or absence of one or more similar or dissimilar other ring substituents; (Y is a halogen or other leaving group) which should effect a similar facile phosphorylation. Furthermore, triesters, derived from oxygen nucleophiles, or any other phosphorylated derivative containing the polyfluoroaromatic phosphate diester protection; wherein Y = -OR,-NRR',-NRR'R'', -SR, (where each group R, R' or R'' can be any suitable moiety as defined above) should be more labile to deprotection under acidic conditions (and/or under basic conditions) than the corresponding bis-phenyl phosphate diester protection.

This method provides higher yields of phosphorylated product of high quality with less or no wasteful side reactions.

This invention is further described in a non-limiting manner by reference to the following examples and accompanying figures wherein:

Fig. 1a Illustrates the enzymatic dephosphorylation of a phosphorylated threonine (or serine) residue.

Fig. 1b Illustrates the enzymatic dephosphorylation of a phosphorylated tyrosine residue.

Fig. 1c Illustrates the enzymatic dephosphorylation of a phosphorylated histidine residue.

Fig. 2: Illustrates reaction schemes 1A & 1B. Reagents and Conditions: i) 20%

piperidine/DMF; ii) 5% $(CH_3CO)_2O$/DMF; iii) DMAP, TEA, $PO(OPh)_2Cl$, DCM, 20°C; iv) 82.5% TFA: 5% phenol: 5% H_2O: 5% thioanisole; 2.5% EDTA (reagent K), 80%; v) LiOH (aq), EtOH/CH_3CN; vi) DMAP, TEA, $PO(OPhF_5)_2Cl$, DCM, 20°C; vii) Dowex Cl, 60%.

Fig. 3a: Shows the structure of bis(pentafluorophenyl) chlorophosphate (11).

Fig. 3b: Shows the structure of the bis(pentafluorophenyl) phosphate derivative of cyclohexanol (12).

Fig. 4: Shows the structure of pentafluorobenzyl-pentafluorophenyl chlorophosphate (13).

Fig. 5: Illustrates reaction scheme 2. Reagents and Conditions: i) 1.01 eq *N*-Chlorosuccinimide, toluene, 2hr, rt: ii) NaH, C_6F_5OH, THF, 1hr, rt; iii) a) NaI, acetone, Δ, 15 mins. b) $HCl_{(aq)}$; iv) PCl_5, DCM.

Fig. 6a: Shows the structure of the benzyl pentafluorophenyl derivative of cyclohexanol (18).

Fig. 6b: Shows the structure of the benzyl pentafluorophenyl derivative of *N*-α-^{t}Boc-tyrosine methyl ester (19).

Fig. 6c: Shows the structure of the phosphopeptide Asp-Ala-Asp-Glu-Tyr(OPO_3H_2)-Leu (23).

Fig. 7: Illustrates reaction scheme 3. Reagents and Conditions: i) 20% piperidine/DMF; ii) DMAP,

TEA, $PO(OCH_2Ph)(OPhF_5)$, DCM, 20°C; iii) NaOH (aq), DMSO; iv) 90% TFA, 5% H_2O, 5% Et_3SiH.

Example 1

Diphenyl chlorophosphate had been successfully employed to phosphorylate the secondary alcohol groups of myo-inositol and its analogues[7]. Using an N-acetyl (Ac) capped analogue of a known consensus sequence for a PP2A substrate as the target, AcNH-Arg-Arg-Ala-Thr(PO_3H_2)-Val-Ala-OH (1), a series of solid-phases phosphorylation reactions were examined. Accordingly, using Wang resin, standard Fmoc chemistry with PyBOP activation, and arginine residue precursors containing 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) protected guanidino groups, the peptide Fmoc-NH-Arg-Arg-Ala-Thr-Val-Ala-O-Wang (2) was prepared. The N-terminal Fmoc group was removed with 20% piperidine in DMF and the free amino group was capped with 5% acetic anhydride in DMF to give compound (3). Treatment of the resin-bound peptide (3) with diphenyl chlorophosphate gave some of the required diphenyl threonine phosphate triester (4), and under optimised conditions (repeated treatments with 20 equivalents of diphenyl chlorophosphate in the presence of DMAP and TEA for 6-8 hours at ambient temperature) essentially quantitative conversion to the triester (4) could be achieved, as determined by NMR-spectroscopic analysis of the products after cleavage from the resin Fig. 2, Scheme 1A.

^1H-, ^{13}C- and ^{31}P-NMR spectra showed the expected signals, chemical shift changes and P-C and P-H couplings for the required triester (5). All attempts to hydrolyse the pure triester (5) under mild basic conditions resulted in the formation of significant

quantities of the β-elimination product, dehydrobutyrine peptide (6), as judged by ^{1}H- and ^{31}P-NMR spectroscopy.

Example 2

In order to increase the electrophilicity at phosphorus in the phosphorylating species (to decrease reaction times) and also in the required peptide phosphate triester (to facilitate deprotection), the preparation and use of bis-(pentafluorophenyl) chlorophosphate (11) was investigated. The reagent was prepared by treating phosphorus oxychloride (7) with 1.8 equivalents of pentafluorophenol (8) at 140°C for 16-24 hours and was purified by removing the unreacted starting materials by distillation. The resulting reagent (11) was 85-90% pure as judged by ^{19}F- and ^{31}P-NMR spectroscopy and could be further purified by fractional distillation.

$$POCl_3 + 2\ C_6HF_5O \longrightarrow (C_6F_5O)PClO + 2\ HCl$$

Example 3

In model reactions using cyclohexanol, the bis-(pentafluorophenyl) chlorophosphate (11) reacted at least 30-fold more rapidly than diphenyl chlorophosphate to give the required triester (12) which was fully characterised. Note that the bis-(2,3,5,6-tetrafluorophenyl) chlorophosphate analogue of reagent (11), which was more useful for mechanistic studies and for product characterisation (due to the presence of an integratable proton resonance in ^{1}H-HMR spectra), behaved similarly in effecting rapid phosphorylation.

Example 4

Treatment of the Pmc protected resin-bound peptide, Ac-NH-Arg-Arg-Ala-Thr-Ala-Val-Ala-O-Wang(3), with 10 equivalents of bis-(pentafluorophenyl) chlorophosphate under optimised conditions gave the resin-bound phosphate triester (9) in excellent yield, Scheme 2B. Immediate deprotection of the two Pmc groups, the two pentafluorophenyl groups, and simultaneous cleavage from the resin occurred upon treatment with aqueous trifluoroacetic acid solutions to give the almost pure N-capped phosphorylated threonine peptide (10) in essentially quantitative conversion. There was no evidence whatsoever for β-elimination products and purification on Dowex 1 chloride (Trade Mark) gave the pure phosphopeptide (10) in 60% overall yield (over 14 solid-phase steps). This material was fully characterised and served as a substrate for protein phosphatase λ as judged by directly monitoring the course of phosphopeptide hydrolysis by ^{1}H-NMR spectroscopy.

Example 5

Other peptides containing serine residues or tyrosine residues were also successfully phosphorylated with bis-(pentafluorophenyl) chlorophosphate (11) using similar protocols.

Example 6

Merrifield resin bound inositol analogues, connected by ether linkages which are stable to trifluoroacetic acid, were successfully phosphorylated on secondary alcohol moieties by bis-(pentafluorophenyl) chlorophosphate (11) using similar protocols.

Treatment with aqueous trifluoroacetic acid resulted in the deprotection of the pentafluorophenyl groups to give resin bound inositol monophosphate analogues. (δp (121.41 MHz, $C_6{}^2H_6$): -10.443).

Example 7

In both solution and solid phase phosphorylations of phenols it was noted that, whilst the actual phosphorylation reaction with bis-(pentafluorophenyl) chlorophosphate (11) was facile, complete removal of the pentafluorophenyl groups was difficult. In each case, the first pentafluorophenyl group could be removed easily in the presence of trifluoroacetic acid solution, but the second pentafluorophenyl group could not. Therefore, since it appeared that the acidity of the partially deprotected phosphoric acid derivative was too high for protonation by the trifluoroacetic acid solution, modified reagents were designed, [for example preferably formula I, where II is a substituted phenyl group (where X, X', X'', X''' are H or F atoms or any suitable moiety), III is a benzyl or substituted benzyl group (where X, X', X'', X''', X'''' are H or F atoms or any suitable moiety) but not a phenyl or substituted phenyl group, and Y is any halogen. It was expected that the phenyl or substituted phenyl group (derived from the reagent) of the intermediate triester (phosphorylated alcohol or phenol) would be removed in a facile manner by base catalysed hydrolysis, and that the benzyl or substituted benzyl group could be removed in a facile manner by acid catalysed hydrolysis, preferably in the presence of trifluoroacetic acid, which would be compatible with other solid state synthesis protocols.

To prepare such substituted phenyl substituted benzyl

halophosphates, a model synthetic protocol was developed using benzyl pentafluorophenyl chlorophospate as the target (Scheme 2).

Treatment of dibenzyl phosphite (14) with *N*-chlorosuccinimide in toluene[8], followed by reaction with sodium pentafluorophenolate (formed by the reaction between sodium hydride and pentafluorophenol in THF) resulted in the formation of dibenzyl pentafluorophenyl phosphate triester (15). ^{1}H, ^{13}C, ^{19}F and ^{31}P-NMR spectra showed the expected chemical shift changes and P-C and P-H coupling constants consistent with those expected for the required triester. δ_H(300 MHz, C^2HCl_3):5.21 (d, J_{PH}8.7, CH_2OP), δ_C(75.4 MHz, C^2HCl_3):70.88 (d, CH_2OP, J_{PC} 6.5), δ_P(121.41 MHz, C^2HCl_3): -5.44, and the correct mass ion (*m/z*(CI+ mode) 444, M^+ molecular ion).

Reaction of the triester (15) with 1 equivalent of anhydrous sodium iodide in refluxing acetone for 15 minutes gave a white solid, which upon cooling was isolated by filtration, then dissolved in water and treated with aqueous hydrochloric acid.[9] The resulting precipitate of benzyl pentafluorophenyl phosphoric acid diester (16) was isolated in essentially quantitative yield from the dibenzyl pentafluorophenyl phosphate triester (15). ^{1}H, ^{13}C, ^{19}F and ^{31}P-NMR spectra showed the expected chemical shift changes and P-C and P-H coupling constants for the required diester. δ_H,(300 MHz, C^2HCl_3):5.20 (2H, d, J_{PH} 8.4, CH_2OP), δ_C(75.4 MHz, C^2HCl_3):70.92 (d, CH_2OP, J_{PC} 5.4), δ_P(121.41 MHz, C^2HCl_3): -4.66. Mass spectrometry confirmed the desired product had been obtained (*m/z* (EI+ mode): 354 (M^+ molecular ion)). Reaction of benzyl pentafluorophenyl phosphoric acid diester (16) with an excess of PCl_5 in dichloromethane followed by removal of the solvent at reduced pressure (20mm/Hg) and separation of the by-

products by distillation at 0.1 mm Hg/30-40°C afforded the reagent (17) in better than 75% purity as judged by ^{1}H and ^{31}P-NMR. δ_H(300 MHz, C^2HCl_3):5.38 (2H, d, J_{PH} 9.9, CH_2OP), δ_C(75.4 MHz, C^2HCl_3):72.91 (d, CH_2OP, J_{PC} 7.54), δ_p(121.41 MHz, C^2HCl_3): main peak at -2.39. Mass spectrometric analysis also gave the expected data (*m/z* (EI+): 372, 374 (Cl isotopes, M^+ molecular ion). The reagent was found to be unstable at high temperatures (50°C) and decomposed if heated for prolonged periods above that temperature. The major contaminant displayed 2 signals at -18.6 and -19.5 ppm in the ^{31}P NMR spectrum of the product and corresponding signals in the ^{1}H, ^{13}C and ^{19}F NMR spectra, consistent with the expected properties of the bis-(benzyl)-bis-(pentafluorophenyl) pyrophosphate. The mass spectrum of the contaminant showed a molecular fragment (*m/z* (CI+) 507, $[M-OPhF_5]^+$) consistent with the structure of the pyrophosphate. Since this material would give identical phosphorylated products to the chlorophosphate, the crude reagent was used routinely for solid phase phosphorylations.

Other benzyl phenyl chlorophosphates were prepared using analogous methods.

Example 8

In model phosphorylation reactions in solution using cyclohexanol, the benzyl pentafluorophenyl chlorophosphate (17) reacted with cyclohexanol in the presence of triethylamine in dichloromethane to give the required triester (18). This was characterised by ^{1}H, ^{13}C, ^{19}F and ^{31}P-NMR spectroscopy and gave the expected data.

Example 9

In model phosphorylation reactions in solution using N-^{t}Boc-(2S)-tyrosine methyl ester, the benzyl pentafluorophenyl chlorophosphate (17) reacted with the phenolic hydroxyl group in the presence of triethylamine in dichloromethane to give the required triester(19). This was characterised by ^1H, ^{13}C, ^{19}F and ^{31}P-NMR spectroscopy and mass spectrometry and gave the expected data.

Example 10

In model solid state phosphorylation reactions, treatment of the resin-bound peptide Fmoc-Val-Tyr-Leu-O-Wang (20) with 10 equivalents of freshly prepared benzyl pentafluorophenyl chlorophosphate (17) under optimised conditions gave the resin bound phosphate triester (21) in excellent yield, Scheme 3. Treatment with 20% piperidine in DMF removed the N-terminal Fmoc group. Subsequent treatment of the product with an excess of 1 $mol.dm^{-3}$ aqueous NaOH in DMSO followed by washing and treatment with aqeuous trifluoroacetic acid resulted in deprotection of the pentafluorophenyl and benzyl groups and cleavage of the resin C-terminal ester linkage to give the phosphopeptide Val-Tyr(OPO_3H_2)-Leu (23), δ_p (121.41 MHz, ^2H_2O):-3.42.

Example 11

Treatment of the *tris*-tert-butyl ester protected resin bound peptide Fmoc-NH-Asp(O^{t}Bu)-Ala-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Tyr-Leu-O-Wang in a similar manner to that described in Example 10 above afforded the almost pure hexapeptide Asp-Ala-Asp-Glu-Tyr(OPO_3H_2)-Leu (24) which showed the expected NMR spectroscopic data. This product

corresponds to the structure of the autophosphorylation site of the epidermal growth factor receptor (EGFR)[10] in its phosphorylated form.

Example 12

Treatment of the resin bound and protected peptide Ac-NH-Arg(Pmc)-Arg(Pmc)-Ala-Thr-Val-Ala-O-Wang (3) with 10 equivalents of benzyl pentafluorophenyl chlorophosphate (17) under optimised conditions gave the benzyl pentafluorophenyl peptide phosphate triester. Treatment of the resulting triester overnight with an excess of 1 $mol.dm^{-3}$ aqueous NaOH in DMSO followed by washing and subsequent treatment with aqueous trifluoroacetic acid resulted in deprotection of the two 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) groups, the pentafluorophenyl and benzyl groups and cleavage of the C-terminal resin ester moiety to give the almost pure N-capped phosphohexapeptide (10). Spectroscopic data showed this material to be identical to that prepared in Example 4 above.

The serine analogue of (10) was prepared using a similar protocol.

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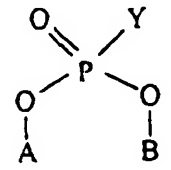
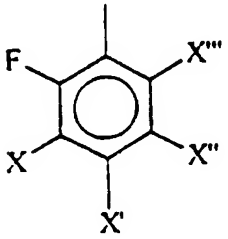
Claims

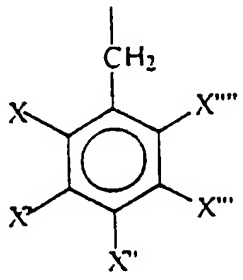
1. An electrophilic phosphorylating reagent for amino acids and/or peptide sequences thereof comprising of a compound represented by formula (I) wherein:



(I)



(II)



(III)

A is a substituted aromatic group which is represented by formula (II) or A is an acid cleavable functionality such as a benzyl or substituted benzyl group represented by formula (III);
B is a substituted aromatic group represented by formula (II); X, X', X'', X''' and X'''' are each H or F atoms or any suitable moiety;
Y is a halogen or leaving group.

2. A phosphorylating reagent as claimed in Claim 1, wherein at least one of the compounds represented by formulae (II) and (III) is fully substituted by fluorine.

3. A phosphorylating reagent as claimed in either of Claims 1 and 2 wherein group Y is a chlorine atom.

4. A phosphorylating reagent as claimed in any one of Claims 1 to 3 which is bis(pentafluorophenyl) chlorophosphate .

5. A phosphorylating reagent as claimed in Claim 3 which is bis (2, 3, 5, 6 - tetrafluorophenyl) chlorophosphate.

6. A phosphorylating reagent as in any one of Claims 1 to 3 where the reagent is a benzyl, fluorophenyl halophosphate.

7. A phosphorylating reagent as in claim 6 where the reagent is a benzyl, polyfluorophenyl chlorophosphate.

8. A method for the phosphorylation of oxygen, nitrogen or sulphur nucleophiles of amino acids and/or compounds comprising an amino acid-like moiety wherein the nucleophile is treated with an excess of a reagent of general formula (I) as defined in Claim 1 followed by the hydrolysis of the product.

9. A method as in claim 8 where the oxygen nucleophile may be part of a primary or secondary alcohol, phenol, carboxylate or enolate group.

10. A method as in either one of claims 8 and 9 where the amino acids may be present as single species or in combination within or outwith the same molecule, as in peptide sequences.

11. A method as in claim 10 where the amino acid(s) may be tyrosine, serine and threonine.

12. A method as in any one of claims 8 to 11 where the amino acid and/or peptide is present as a resin bound moiety.

13. A method as in any one of claims 8 to 12 where the phosphorylation method may be utilised in solid, liquid or gel phase.

14. A method as in any one of claims 8 to 13 where the hydrolysis reagent is trifluoroacetic acid.

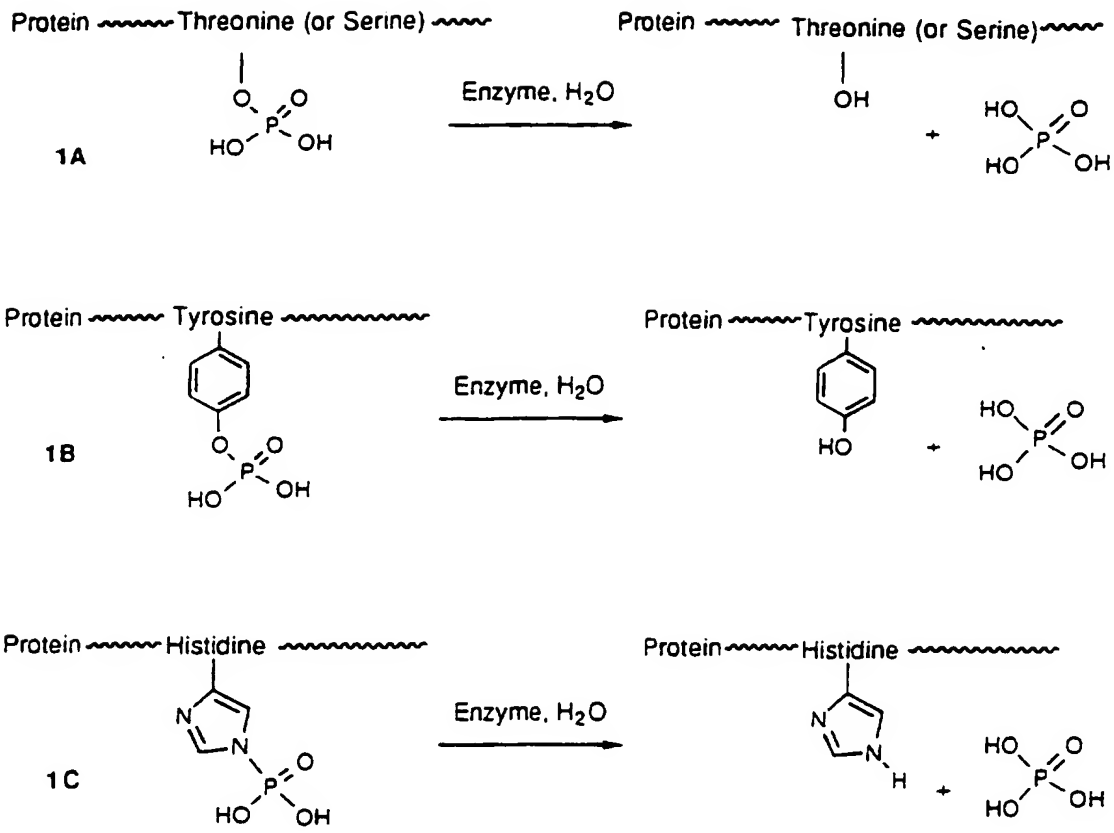


FIGURE 1

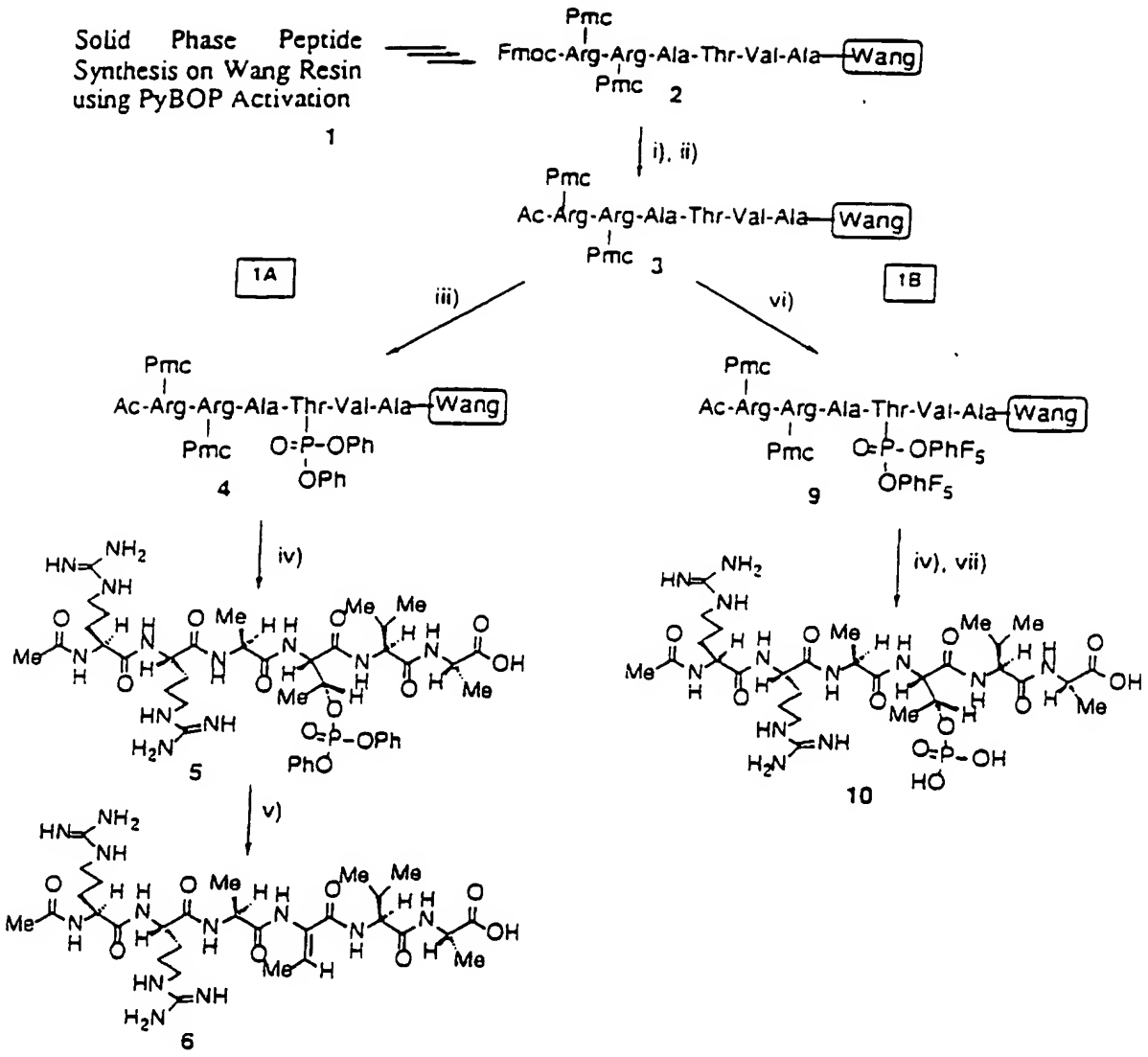


FIGURE 2

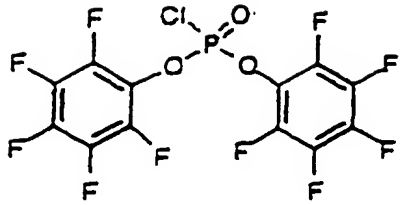


FIGURE 3A

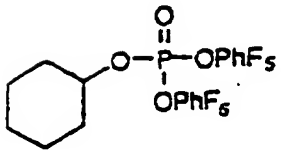


FIGURE 3B

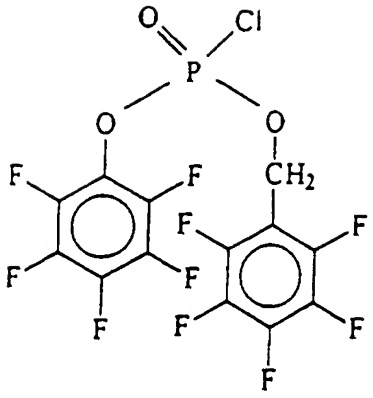


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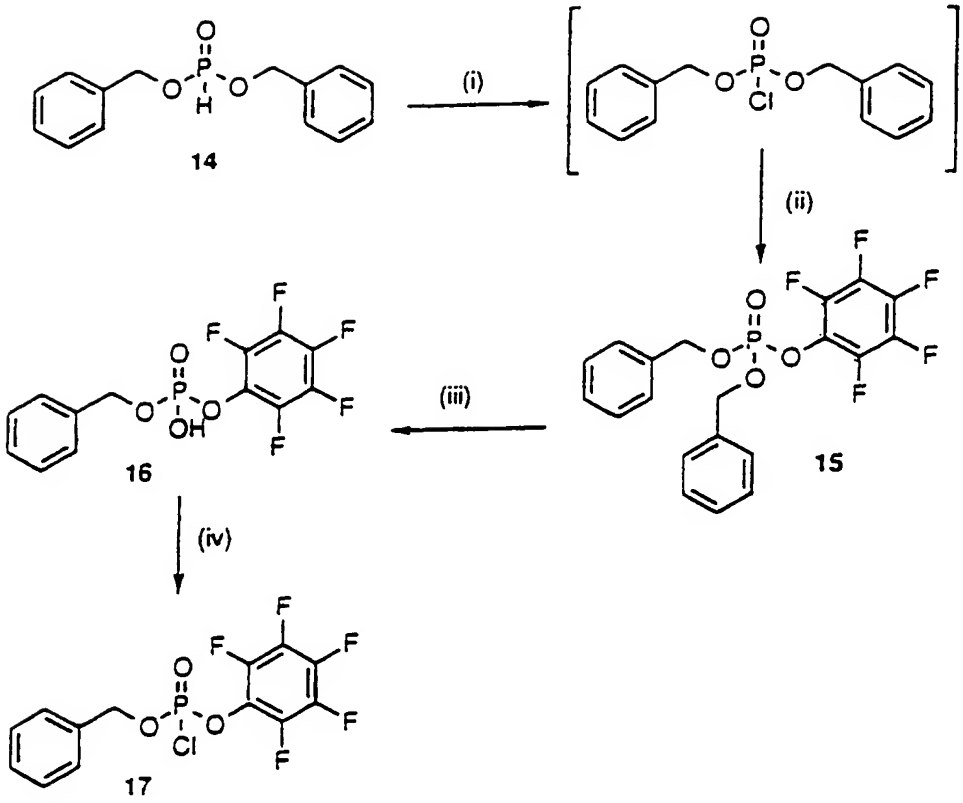
FIGURE 4

FIGURE 5



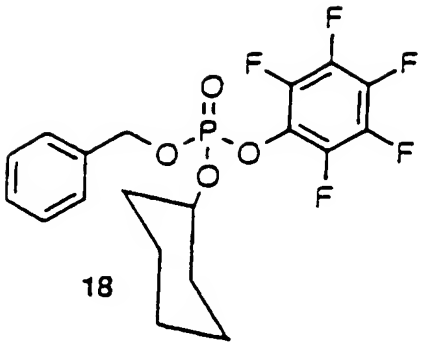


FIGURE 6A

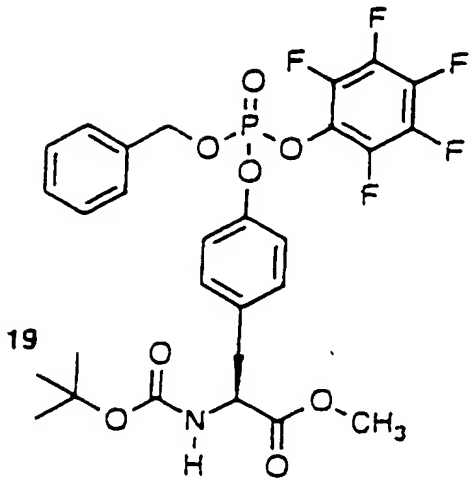


FIGURE 6B

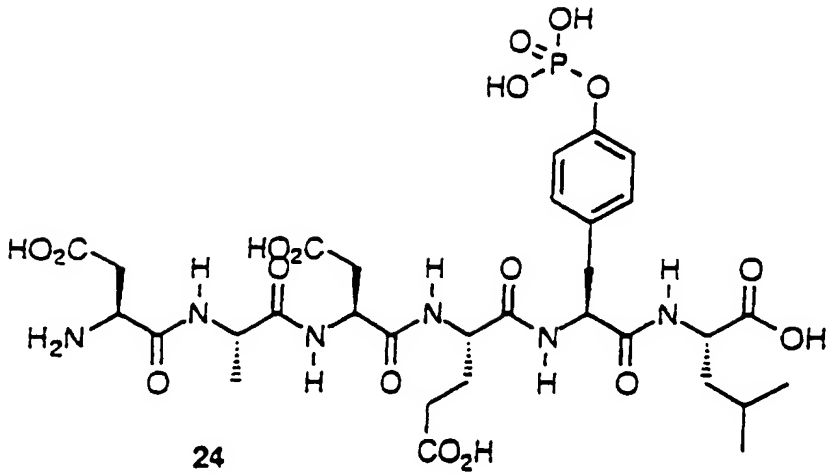


FIGURE 6C

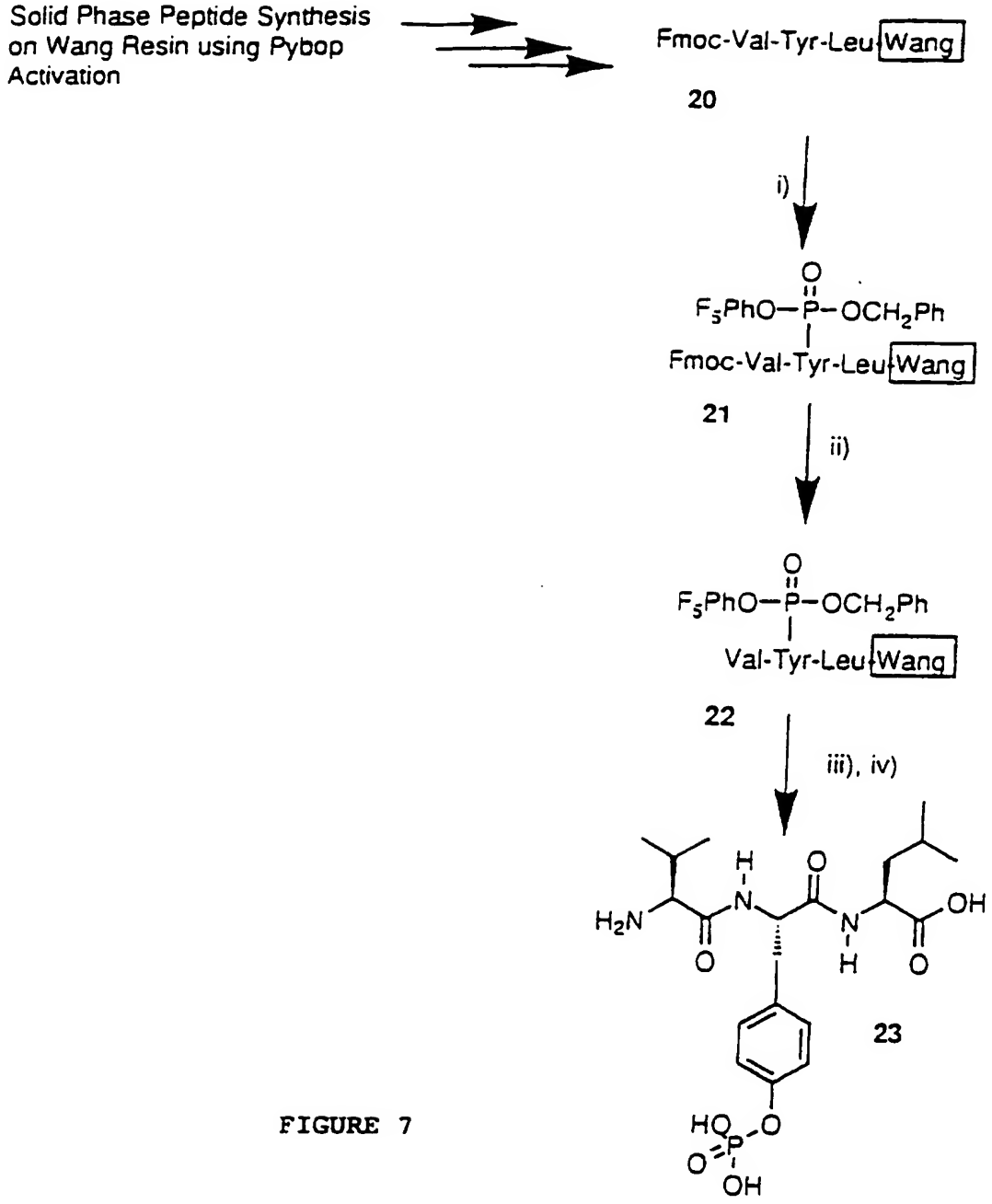


FIGURE 7

INTERNATIONAL SEARCH REPORT

Inte inal Application No
PCT/GB 97/02592

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07F9/14 C07K1/00 C07K1/04 C07F9/12

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07F C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3 341 630 A (ROBERT H. BOSCHAN) 12 September 1967 see the whole document ---	1-4
X	US 3 341 631 A (CHRISTIAN A. SEIL) 12 September 1967 see the whole document ---	1,2
X	US 3 408 427 A (ROBERT H. BOSCHAN) 29 October 1968 see the whole document ---	1,3
Y	US 5 245 069 A (JAMES W. MCMANUS) 14 September 1993 see the whole document --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 November 1997	04/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Beslier, L

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INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/GB 97/02592

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHULZ J ET AL: "Synthesis and properties of mechanism-based inhibitors and probes for inositol monophosphatase derived from 6-O-(2'-hydroxyethyl)-(1R,2R,4R,6R)-cycloh exane-1,2,4,6-tetraol" J. CHEM. SOC., CHEM. COMMUN. (JCCCAT,00224936);95; (22); PP.2353-6, THE UNIVERSITY, ST. ANDREWS;SCHOOL CHEMISTRY; FIFE; KY16 9ST; UK (GB), XP002047053 cited in the application see the whole document	1-14
Y	JAN HES: "Di(2-tert-butylphenyl) Phosphorochloridate. A new selective Phosphorylating agent." JOURNAL OF ORGANIC CHEMISTRY., vol. 39, no. 25, 1974, EASTON US, pages 3767-3769, XP002047054 see the whole document	1-14
P,X	HORMOZDIARI P ET AL: "Highly efficient solid-phase phosphopeptide synthesis using bis(polyfluorophenyl) chlorophosphates: preparation of serine-threonine protein phosphatase substrates" TETRAHEDRON LETT. (TELEAY,00404039);96; VOL.37 (45); PP.8227-8230, THE UNIVERSITY;SCH. CHEM.; ST. ANDREWS, FIFE; KY16 9ST; UK (GB), XP002047055 see the whole document	1-14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/02592

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 3341630 A	12-09-67	NONE	
US 3341631 A	12-09-67	NONE	
US 3408427 A	29-10-68	NONE	
US 5245069 A	14-09-93	NONE	